Copper Limitation with Penicillamine

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In work with soft tissue calcification in cattle and sheep occurring under field conditions it was noted that frequently, if not always, analysis of liver showed very low levels of copper (Bingley and Carrillo, 1966). It has been of interest that the elastic fibers which are the site of early calcification in the aorta are also the site of degeneration under copper deficiency conditions.

Efforts to develop a satisfactory laboratory animal model to study this soft tissue calcification suggested the possible need for a low copper diet, especially if very low levels of the causative agent were to be used. The guinea pig as an herbivorous animal, known to be susceptible to soft tissue calcification, was chosen for our studies.

The initial problem was to develop a satisfactory, palatable, low copper diet. The commercially available low copper diets retained sufficient copper to meet minimum needs of the animals. Our attempts at preparing such a diet faltered on the problem of palatability.

We obtained a pelleted low copper Reid-Briggs diet from Nutritional Biochemical Co., Cleveland, Ohio. It consisted of vitamin free casein, alphacel, sucrose, corn oil, and a copper free mineral mixture. Analysis by Bechman Atomic Absorption Spectrometer indicated 4 ppm Cu, significantly higher than our goal of 1 ppm Cu.

The difficulties experienced with the production or purchasing of a palatable low copper diet suggested to us the possibility of introducing a chelating agent which might reduce utilization or produce a negative balance causing a functional if not an absolute deficiency.

Since studies with human patients with Wilson's disease and with normal subjects showed that penicillamine increased urinary copper (Walshe, 1956, 1956a, 1964; Scheinberg and Sternlieb, 1960) and decreased the concentration of copper in the liver (Scheinberg and Sternlieb, 1960, 1963) this compound was selected for study with guinea pigs. The present report is concerned with the results

of our efforts to ascertain the influence of orally administered penicillamine upon storage of body copper. Our eventual goal is to produce a guinea pig with low copper reserves that may be used in studies of cardiovascular calcification.

Experimental Procedure

Young male Hartley strain guinea pigs were purchased from Simonsen Lab. Inc., White Bear, Minnesota, and were caged into stainless steel cages with stainless steel wire floors. The metabolic pans were also made from stainless steel. Purina Guinea Pig Chow was fed during the experiment and contained 11.66 ppm of copper as analyzed by the Atomic Absorption Spectrophotometer. D-penicillamine was purchased from Nutritional Biochemical Co. and used in the amount of 150 mg per day administered orally for a period of 10 days. Daily (24 hours) collection of urine was performed before penicillamine treatment for a period of 4 days and during penicillamine treatment for 10 days. Total fecal collection was done one time before and 3 times during penicillamine treatment.

The urine was diluted one to one with trichloroacetic acid, 10 per cent, filtered, and stored in the refrigerator for further determination. Feed, fecal, and tissue samples were first dried and later ashed in a furnace at 600 C for 16 hours. The ashed samples were digested for one hour on a hot plate with 50 per cent concentrated HCl, filtered, and made up to volume with triple distilled water.

The animals were killed 24 hours after the last dose of d-penicillamine and blood, liver, kidney, and spleen were collected.

Hemoglobin was determined by the cyanmethemoglobin method with a Bausch & Lomb Spectronic 20. Copper determinations in plasma, feed, urine, fecal matter, and organs were performed with the Perkin Elmer 303 Atomic Absorption Spectrophotometer.

Results

In initial tests there was a high mortality rate with guinea pigs receiving 150 mg dl-penicillamine orally, which is apparently more toxic for the guinea pigs than the d-penicillamine, and it was decided to use the latter in this experiment.

The effect of d-penicillamine, upon the excretion of copper in the urine during a ten day experimental period was significantly higher ($P<.01$) than the excretion before the penicillamine was administered to the animals (Table 1).

TABLE 1
Effect of d-penicillamine on copper retention in guinea pigs

Treatment	Pretreated (4 days)		Treated (10 days)	
Experiment No.	1	2	1	2
No. of Animals	8	8	8	8
Intake (mg)	264.9	320.4	243.9	285.3
Fecal (mg)	228.1	281.2	205.3	245.0
Absorbed (mg)	36.8	39.2	38.6	40.3
Absorbed (% of intake)	13.9	12.2	15.8	14.1
Urinary (mg)	21.0	22.7	46.0	44.4
Urinary (% of intake)	7.9	7.1	18.9	15.6
Retention (mg)	15.8	16.5	–7.4	–4.1
Retention (% of intake)	6.0	5.1	–3.1	–1.5
Retention (% of absorption)	42.9	42.1	–19.4	–10.2

The increased excretion of copper in urine started within 24 hours and reached the highest level on the 5th and 8th day.

In order to see the after effect of penicillamine on the urinary copper excretion a second experiment was conducted. The urine copper was determined for 10 days after the last dose of penicillamine administration (Fig. 1).

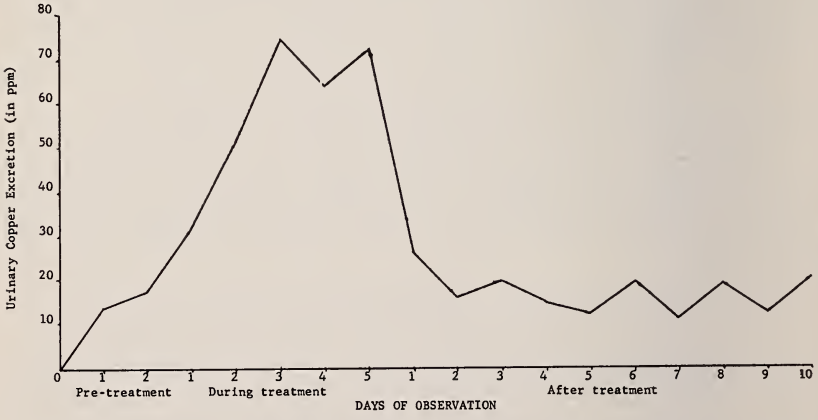

Fig. 1. Urinary copper excretion before, during, and after treatment with d-penicillamine.

It appeared that urinary copper excretion reached a normal level within 48 hours after the last dose of penicillamine intake.

All the treated animals, except two (one in each experiment), showed a negative copper retention caused by penicillamine treatment. It is also possible that penicillamine had an effect on the absorption of dietary copper. Animals receiving penicillamine had a higher per cent absorption rate than the pretreated ones. However, in these experiments the difference was not statistically significant (Table 1).

The negative retention of copper due to penicillamine administration was reflected in a significant decrease in copper storage in liver and spleen, but not in kidney.

The two animals on penicillamine treatment that showed a positive retention had an amount of copper in liver and spleen similar to that of the controls and both were significantly higher than those in animals which had negative retention (Table 2).

TABLE 2

The effect of d-penicillamine on copper storage

	Liver		Spleen		Kidney	
	μg/gm dry weight	μg total	μg/gm dry weight	μg total	μg/gm dry weight	μg total
Treatment						
Normal	96.5	640°°	39.0	8.80°	77.6	71.0n.s.
Treated	54.5	280	31.2	5.34	61.5	65.4

° $P<.05$
°° $P<.01$
n.s. not significant

There were no significant differences in the plasma copper, hemoglobin content, and hematocrit between the treated animals 24 hours after the last dose of penicillamine and the controls.

Discussion

In the preliminary trials we tried to produce a very low copper diet (<1 ppm) that was palatable for guinea pigs. Special feed samples from General Biochemicals Corp., Chagrin Falls, Ohio, and Nutritional Biochemicals Co. of Reid-Briggs formula for guinea

pigs were also purchased and analyzed. The diet that contained purified casein, sucrose, alphacel, corn oil, and copper free vitamin mineral mixture was given to guinea pigs after gradually changing from the Purina Chow. The pelleted purified feed was less palatable and produced diarrhea. The copper content of this diet was determined by both the Perkin Elmer and Beckman Atomic Absorption Spectrophotometers and ranged between 2 to 4 ppm. Although we kept these animals on this low diet for more than 3 months, they continued to gain weight and did not show signs of Cu deficiency. Everson et al. (1967) succeeded in producing a laboratory guinea pig diet which contained 0.5 and 0.7 ppm copper. They used non fat dry milk solid, glucose (cerelose), EDTA treated alpha-cellulose, cotton seed oil, salts, and vitamin mineral mixture in the feed and the copper content was determined by the carbamate colorimetric method. We elected to pursue a different technique.

Due to these difficulties in securing a diet which contained copper less than 1 ppm as determined by atomic absorption spectrophotometry, the penicillamine treatment was used to produce animals with a low copper storage in the body.

Experiments conducted in rats have shown that dl and l-penicillamine are toxic and the LD 50 per oral administration has been found to be 365 mg/kg body weight (Aposhian and Aposhian, 1959; Wilson and du Vigneaud, 1950). This toxicity was attributed to the involvement of dl-penicillamine with the pyridoxal-5-phosphate and the increased pyridoxine excretion in urine. Dl-penicillamine was shown in our experiments to be toxic for guinea pigs when they received orally about 320 mg/kg of body weight. However, d-penicillamine was not toxic to guinea pigs at a dose of 400 mg per kg of body weight, although the body weight gain was lower during the 10 days treatment.

Walshe (1964) in his clearance studies in Wilson's disease patients supports his hypothesis that penicillamine depletes the body stores of copper. Direct evidence for a fall in the concentration of copper in liver was shown by Scheinberg and Sternlieb (1960). Walshe (1964) suggests the probable immediate action of penicillamine is to render the plasma copper available for filtration at the glomerulus by breaking the copper-albumin linkages.

In this experiment the increased urinary copper during the

ten days caused a significant decrease of copper storage in the liver and spleen, but not in the kidney. The copper present in the glomeruli and tubules of the kidney, perhaps in process of excretion may have been the cause of the nonsignificant difference in the copper content between the treated animals and the controls.

It is our belief that penicillamine given to guinea pigs for a longer period may cause a depletion of the body copper stores and that these animals can then be utilized in experiments requiring low copper reserves.

SUMMARY

Guinea pigs treated with d-penicillamine orally for 10 days excreted in their urine 2 to 4 times the copper found in the urine of the controls. The d-penicillamine was not toxic as compared to the dl-penicillamine at a level of approximately 400 mg per kg body weight. The copper storage in liver and spleen was significantly decreased.

This treatment may be used as the alternative of feeding low copper diet in conditioning guinea pigs for further experiments to study the relationship of copper deficiency to soft tissue calcification.

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